THE PURIFICATION OF RESTRICTION ENDONUCLEASE EcoRI BY PRECIPITATION INVOLVING POLYETHYLENEIMINE

Alistair H. A. BINGHAM, Andrew F. SHARMAN and Tony ATKINSON
Microbiological Research Establishment, Porton, Salisbury, Wiltshire, England

Received 9 February 1977

1. Introduction

The restriction endonuclease EcoRI from an *Escherichia coli* containing the plasmid pMB 1, 3 or 4 [1] is widely used in the analysis and manipulation of DNA molecules. The enzyme has been used in the physical mapping of viral genomes [2,3] and mitochondrial DNA [4], the preliminary analysis of DNA molecules [5–7] and since cleavage with EcoRI yields cohesive terminii, in the in vitro construction of recombinant DNA molecules [8].

Homogeneous enzyme preparations are not essential for the in vitro analysis and manipulation of DNA molecules; a preparation completely free of contaminating non-specific nucleases is adequate for most experiments. There are several published procedures for the purification of EcoRI [9–12], however they all suffer from two major disadvantages, the large number of steps involved in the preparation of exonuclease-free material and the poor yields of the enzyme finally obtained.

The use of polyethyleneimine (PEI) for the removal of nucleic acids from microbial extracts was demonstrated by Atkinson and Jack [13], and it was found that if this procedure was carried out under conditions of low ionic strength with an extract of *E. coli* RY13 the EcoRI activity precipitated with the PEI-DNA complex.

This paper describes a rapid, two step method for the purification of exonuclease-free EcoRI restriction endonuclease in high yield, based on elution of the enzyme from a PEI precipitated DNA–enzyme complex. The preparation of other restriction endonucleases using a similar technique is also discussed.

2. Materials and methods

2.1. *Materials*

Ethidium bromide, lysozyme and phenylmethylsulphonyl fluoride (PMSF), an alkaline protease inhibitor were purchased from Sigma Ltd, London, phosphocellulose, P11 from Whatman Ltd, Maidstone, England, agarose and Bio-beads SM-2 from BioRad Ltd, Bromley, England and PM 10 membranes from Amicon, High Wycombe, England. All other chemicals were purchased from BDH Chemicals Ltd., Poole, England.

De-ionised water autoclaved at 121°C for 40 min to remove traces of protease and nuclease was used for all buffers. Phosphocellulose was activated and equilibrated as described by Greene, Betlach, Goodman and Boyer [9].

PEI (BDH Chemicals) supplied as a 50% solution and diluted to 10% (v/v) with de-ionised water and the pH adjusted to 7.5 with 10 M HCl. Prior to use the solution was dialysed for 24 h against two changes of 25 mM Tris–HCl, pH 7.5, at 4°C and diluted to 2.5% (v/v) with the same buffer.

An 0.8% (w/v) solution of agarose in 90 mM Tris, 90 mM boric acid, 3 mM Na_2.EDTA and containing 1 μg/ml ethidium bromide was autoclaved for 15 min at 121°C to obtain a homogeneous gel.

E. coli RY13 and *E. coli* W3110 (lambda C1857 ts⁻ S7 am) cell-pastes were kindly provided by M. J. Comer, grown according to the method of Bingham, Comer, Sharp and Atkinson (manuscript in preparation). Lambda-DNA was prepared by a modification of the procedure of Thomas and Davis [10].

 North-Holland Publishing Company – Amsterdam

2.2. *Methods*

Protein was estimated by the method of Lowry [14] after removal of Triton X-100 from the samples as described by Holloway [15]. DNA was estimated by the method of Burton [16] and RNA as described by Millitzer [17].

EcoRI restriction endonuclease was assayed by dilution to extinction in 25 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 1 mg/ml gelatin, 0.2% Triton X-100, 10% glycerol and the smallest quantity of enzyme that would completely digest 1 μg lambda-DNA in one hour at 37°C was determined. Incubations were carried out at 37°C by the addition of 10 μl diluted enzyme to 10 μl of 100 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 10 μl of lambda-DNA solution containing 1 μg lambda in 10 mM Tris–HCl, pH 7.4, 1 mM Na_2.EDTA. The reaction was stopped by the addition of 5 μl of 5% SDS, 0.025% bromophenol blue. Electrophoresis was carried out on 0.8% agarose slab-gels (20 × 14 cm) at 75 V for 2–3 h. Gels were visualised on an ultra-violet mineralight transluminator (Ultra-violet Products Ltd, Winchester, England) at 254 nm. The lowest level of visual detection in this system is 5 ng DNA.

The level of contaminating non-specific nucleases present in crude extracts of *E. coli* RY13 was estimated in order to correct the observed, but elevated level of EcoRI in crude extracts, as determined by the above assay. This was achieved by measurement of nuclease activity in extracts of a r^-m^- deletion mutant of *E. coli* RY13 lacking the pMB 3 plasmid, and a two-fold correction was found to be necessary.

Extracts of *E. coli* RY13 were prepared by suspending 50 g cell-paste in 200 ml 25 mM Tris–HCl, pH 8.0, 25 mM NaCl, 1 mM Na_2.EDTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF and the temperature raised to 20°C, 10 ml lysozyme (10 mg/ml) was then added and after 10 min incubation 30 ml 50 mM $MgSO_4$, 5% Triton X-100 was added. The temperature was rapidly reduced to 4°C and lysis completed by 3 × 1 min sonication (20 kHz, 5 A) while maintaining the temperature below 10°C. Cell debris was removed by centrifugation at 25 000 × *g* for 60 min at 4°C and the supernatant adjusted to pH 7.4.

Unless otherwise stated PEI (2.5% solution) was added slowly to the extract (200 ml) to a final concentration of 0.1% (8 ml). After gently stirring for 10 min on ice, the precipitate was collected by centrifugation at 25 000 × *g* for 30 min at 4°C.

The pellet was suspended in 200 ml 25 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM PMSF with a homogeniser (Nelco Ltd., Shalford, Surrey, England). The suspension was centrifuged and the resulting pellet washed twice with 100 ml of the above buffer containing 300 mM NaCl and then twice with 100 ml buffer containing 700 mM NaCl.

The active fractions (300 mM NaCl washes) were pooled and concentrated to half-volume (100 ml) with an Amicon PM 10 ultrafiltration membrane, then diluted with 20 mM potassium phosphate, pH 7.4, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM PMSF, to a conductivity equal to that of 20 mM potassium phosphate, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM PMSF (20 mS). Phosphocellulose (20 g wet wt = 6 g dry wt) equilibrated in the same buffer was added and the suspension stirred for 16 h at 4°C. The resin was allowed to settle and collected into a 1.5 × 25 cm column. The column was washed with one column volume (50 ml) of 20 mM phosphate buffer, pH 7.4, 250 mM NaCl, 0.2% Triton X-100, 10 mM β-mercaptoethanol, 0.1 mM PMSF, and eluted with a 250 ml linear-gradient of 250–750 mM NaCl in the above buffer at 15 ml/h (4°C). Fractions (6 ml) were collected and the active fractions pooled and concentrated to 10–15 ml with an Amicon PM 10 membrane. Gelatin (10 mg/ml) was added to the concentrated pool to give a final concentration of 0.5 mg/ml and the enzyme was then dialysed for 24 h at 4°C against 20 vol. 25 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.5 mM dithiothreitol, 1 mg/ml gelatin, 0.2% Triton X-100, 50% glycerol. The enzyme was stored at −20°C and no loss of activity was observed after 1 year.

3. Results and discussion

Studies on the precipitation of nucleic acids in *E. coli* RY13 extracts by PEI in the presence of varying NaCl concentrations clearly showed that EcoRI precipitated at relatively low ionic-strength (table 1). At high ionic strength, the enzyme remained in the

Table 1
Precipitation of nucleic acids and EcoRI with polyethyleneimine

	Supernatant			
	DNA (total mg)	RNA	Protein	Enzyme (%)
Extract	290	760	3500	100
PEI precipitation in:				
(1) 25 mM NaCl	18	140	2000	0.1
(2) 100 mM NaCl	18	135	2030	28
(3) 300 mM NaCl	19	140	2100	100

An extract of *E. coli* RY13 (50 g) containing various NaCl concentrations was treated with PEI to 0.1% as described in Methods, and the resulting precipitate removed by centrifugation. The level of DNA, RNA, protein and enzyme in the supernatants was determined.

supernatant even though all the DNA was still precipitated by PEI.

The amount of PEI necessary to precipitate EcoRI was investigated in order to reduce the presence of excess PEI in the supernatant. This can interfere in the later stages of the purification due to its affinity for phosphocellulose. The results in table 2 show that 0.1% was in fact optimum for the precipitation of nucleic acids and EcoRI.

The results of table 3 show that EcoRI is eluted from the precipitate, mainly in the 300 mM NaCl wash, with an overall recovery of approximately 70%. A twenty-fold purification was achieved with this single-step and the amount of nucleic acid eluted from the precipitate was within acceptable limits. Observation of assay gels at all stages showed that approximately 25% of the contaminating nucleases did not precipitate while the majority of the remaining exonuclease that precipitated with the PEI–DNA complex elutes in the 100 mM NaCl wash.

The resulting pool of 300 mM NaCl washes contained only a small amount of non-specific nuclease and this was removed by phosphocellulose chromatography (fig.1). Restriction endonuclease EcoRI elutes

Table 2
Effect of concentration of polyethyleneimine on the precipitation of nucleic acids and EcoRI

	Supernatants			
	DNA (total mg)	RNA	Protein	Enzyme (%)
Extract	300	785	3650	100
PEI concentration:				
(1) 0.005%	260	700	3200	100
(2) 0.025%	184	560	2900	95
(3) 0.05%	52	325	2400	46
(4) 0.1%	18	152	2100	< 0.1
(5) 0.2%	15	91	2150	< 0.1

An extract of *E. coli* RY13 (50 g) prepared as described in Methods, was divided into five 40 ml quantities and treated with various concentrations of PEI and the resulting precipitate removed by centrifugation. The levels of DNA, RNA, protein and enzyme in the supernatants were determined. Figures for post-PEI data are equated to a 50 g extraction.

Table 3
Elution of EcoRI from the polyethyleneimine precipitate

		Supernatant					
		DNA (total mg)	RNA	Protein	Enzyme (total U X 10^{-6})	(%)	Specific activity (U/mg protein)
Extract		280	720	3600	5.1	100	1400
100 mM NaCl wash		2.1	22	248	0	0	–
300 mM NaCl wash	(a)	1.6	23	85	2.4	39	28 400
	(b)	1.2	20	66	1.2	17	18 200
700 mM NaCl wash	(a)	1.6	50	80	0.4	10	5000
	(b)	2.0	47	74	0.1	6	1300

An extract of *E. coli* RY13 (50 g cell-paste) was treated with PEI to a final concentration of 0.1% and the resulting precipitate was washed sequentially with buffer containing 100, 300 and 700 mM NaCl as described in Methods. DNA, RNA, protein and EcoRI levels in each of the washes were determined. 1 Unit EcoRI is defined as 1 μg lambda-DNA completely digested in 1 h at 37°C.

at approximately 0.55 M NaCl (peak) in this system and after both exonuclease and the major protein peak. Excess PEI, if present in large amounts, binds avidly to phosphocellulose thus reducing the capacity of the column for EcoRI, but elutes at 0.65 M NaCl and therefore is well retarded after the peak of enzyme activity, whilst any PEI–DNA (or PEI–DNA oligonucleotide) complex in the post-NaCl wash from the PEI–DNA–enzyme precipitate elutes from phosphocellulose at 0.35–0.45 M NaCl, well before the peak of EcoRI activity. The phosphocellulose chromatography gives about a 7-fold purification with an overall yield of enzyme of about 50% and a total purification of 150-fold at this stage (table 4). The

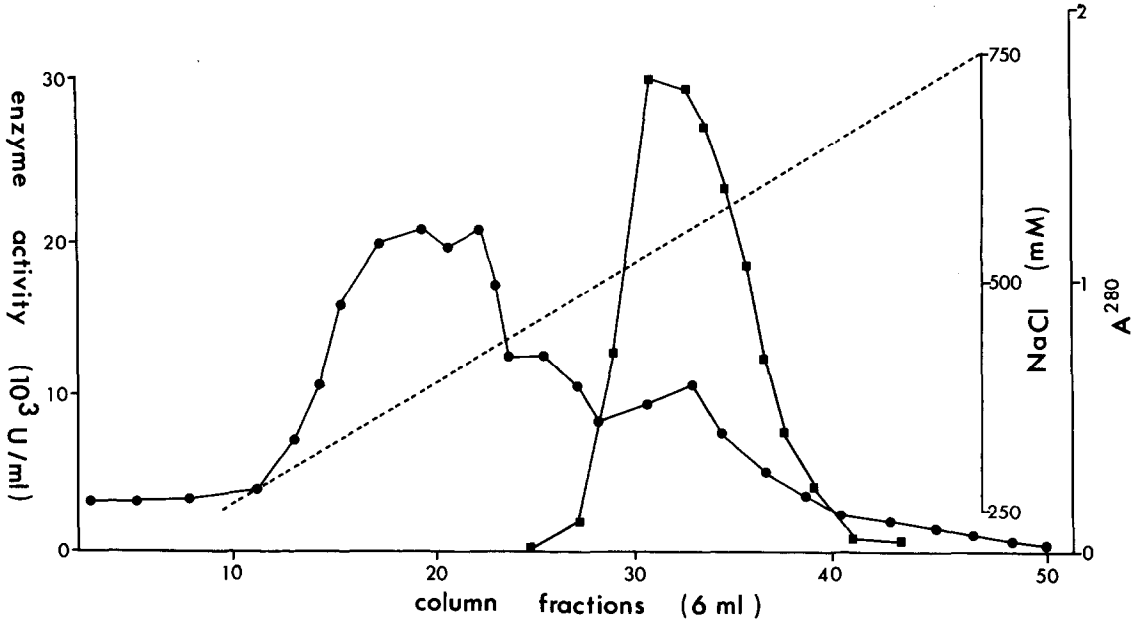

Fig.1. Elution of EcoRI from phosphocellulose with a linear-gradient of 250–750 mM NaCl (- - - - -) as described in Methods. (●———●) Elution of protein as determined by absorbance at 280 nm. (■———■) EcoRI activity, 1 unit is defined as 1 μg lambda-DNA completely digested in 1 h at 37°C.

Table 4
Summary of the purification of EcoRI restriction endonuclease from 50 g cell-paste

	Protein (mg)	Enzyme (total U × 10^{-6})	(%)	Specific activity (U/mg)
Extract	3600	5.1	100	1400
PEI eluate pool	135	3.6	71	26 600
Phosphocellulose pooled peak	14	2.55	50	182 000

An extract *E. coli* RY13 (50 g cell-paste) was purified as described in Methods protein and enzyme levels were determined at each stage. One unit of enzyme is defined as 1 μg lambda-DNA completely digested in 1 h at 37°C.

specific activity of the final product is 160 000–200 000 U/mg protein compared to 1400 in crude extracts, which represents a substantial purification even though homogenous enzyme has a specific activity of about 3×10^6 U/mg (Bingham et al. manuscript in preparation). By pooling tightly from the phosphocellulose column it is possible to achieve specific activities of 250 000–300 000 U/mg but with a substantial reduction in yield, however, this is not essential for the preparation of exonuclease free EcoRI.

SDS–polyacrylamide gels of the final EcoRI preparation showed that contaminating protein was reduced to 2 major species (fig.2).

The purification procedure for EcoRI described in this paper is of a similar nature to the DNA-dependent RNA polymerase purification of Jendrisak and Burgess [18,19] involving PEI precipitation of the enzyme and giving similar high yields and purification factors despite the comparatively small amount (cf. polymerase) of EcoRI present in the starting material. Jendrisak and Burgess [18,19] found it necessary to shear the viscous DNA in the cell-extract prior to the addition of PEI in order to facilitate precipitation of the PEI–DNA complex. The data in this paper confirm this point and therefore mild sonication of the cell extract is a necessary prerequisite to precipitation.

Evidence that EcoRI precipitates bound to DNA in a PEI–DNA–EcoRI complex rather than as a PEI–EcoRI complex can be inferred from the fact that

(i) EcoRI elution from the PEI–DNA precipitate occurs at about the same ionic strength as elution of the enzyme from a DNA–cellulose matrix (unpublished observations).

(ii) The presence of PEI does not influence the elution position of EcoRI from either phosphocellulose or DNA–cellulose (whereas even low levels of DNA dramatically affect binding and elution.

(iii) In the absence of DNA or oligonucleotides EcoRI elutes from DEAE–cellulose at 0.1 M NaCl, pH 7.5, whereas in their presence a small but significant amount of enzyme elutes at 0.65 M NaCl, pH 7.5, together with DNA fragments (unpublished observations). This phenomenon occurs with *E. coli* RY13 chromosomal DNA and may be evidence that methylation of the DNA by the modification methylase does not prevent binding of the restriction endonuclease.

The possibility that this procedure of PEI precipitation would be applicable for most DNA-binding enzymes, e.g., DNA-ligase, DNA-polymerase as well as other restriction endonucleases has not been investigated fully. However, data presented in this paper show that binding of contaminating nucleases to a PEI–DNA complex is weak and work in progress with plasmid mediated EcoRII (*E. coli* RY23) indicates that this technique, followed by phosphocellose chromatography is satisfactory for the preparation of an exonuclease-free enzyme. Preliminary investigations with the restriction endonucleases Taq 1 (*Thermus aquaticus*), Bst I (*Bacillus stearothermophilus* NCA 1503) and Bcl I (*Bacillus caldolyticus*) indicates that this method of purification does not work as well for these enzymes. This may in part be due to the relatively low level of these enzymes present in the cell in comparison with the plasmid controlled EcoRI, where the presence of

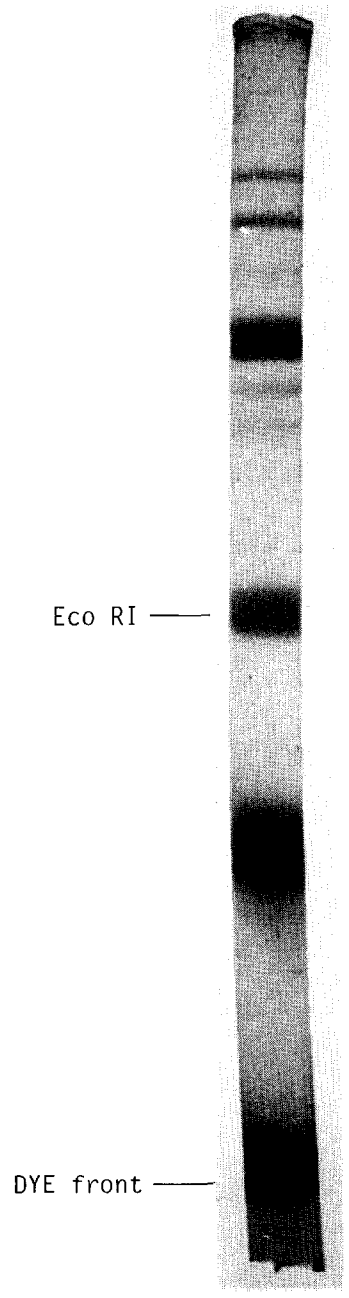

Fig.2. SDS–Polyacrylamide gel of the pooled EcoRI activity from the phosphocellulose column. A sample (50 μl containing 0.5–1 mg/ml protein) was run on 10% SDS–polyacrylamide gels (6 mm × 10 cm) at 0.5 mA/gel for 16 h according to the method of Laemmli [20]. Gels were stained for protein in 0.25% Coomassie Brilliant Blue R stain (Sigma Ltd, London) in 50% methanol/10% acetic acid (2 1) and then washed repeatedly in 25% methanol/10% acetic acid.

multiple copies of the ρMB3 plasmid in *E. coli* RY13 leads to abnormally high enzyme levels [1].

An alternative procedure for the purification of restriction endonucleases that do not precipitate or elute from the PEI–DNA complex would be to use PEI for the removal of nucleic acids under high ionic strength conditions prior to further chromatographic purification (see table 1). This alternative method has proved useful as a first step in the purification of the enzymes from *Haemophilus influenzae* Rd, *Haemophilus parainfluenzae, Serratia marcescens, Xanthomonas badrii* and *Xanthomonas amaranthicola* replacing streptomycin sulphate precipitation of nucleic acids (cf. ref [13]). We have also used this method for the large-scale (25 kg cell-paste) purification of EcoRI to homogeneity (Bingham et al. manuscript in preparation), where the large size of a PEI precipitate leads to practical difficulties in the elution of the enzyme.

The technique of co-precipitation of EcoRI and DNA with PEI, described in this paper, circumvents many of the problems associated with the multi-step purifications of exonuclease-free EcoRI already presented in the literature [9–12]. The validity of this purification procedure resides in the fact that it is rapid, simple to carry out and gives a high yield of exonuclease-free restriction endonuclease for in vitro genetic analysis and manipulation of DNA molecules.

Acknowledgements

The authors would like to thank Mr W. Geraghty for DNA and RNA estimations.

References

[1] Betlach, M., Hershfield, V., Chow, L., Brown, W., Goodman, H. M. and Boyer, H. W. (1976) Fed. Proc. 35, 2037–2043.
[2] Danna, K. J., Sack, G. H. and Nathans, D. (1973) J. Mol. Biol. 78, 363–376.
[3] Khoury, G., Martin, M. A., Lee, T. N. H., Danna, K. J. and Nathans, D. (1973) J. Mol. Biol. 78, 377–389.
[4] Brown, W. M. and Vinograd, J. (1974) Proc. Natl. Acad. Sci. USA 71, 4617–4621.
[5] Streeck, R. E., Philippsen, P. and Zachau, H. G. (1974) Eur. J. Biochem. 45, 489–499.
[6] Firtel, R. A., Cockburn, A., Frankel, G. and Hershfield, V. (1976) J. Mol. Biol. 102, 831–852.

[7] Ito, J., Kawamura, F. and Yanofsky, S. (1976) Virology 70, 37–51.
[8] Mertz, J. E. and Davis, R. W. (1972) Proc. Natl. Acad. Sci. USA 69, 3370–3374.
[9] Greene, P. J., Betlach, M., Goodman, H. M. and Boyer, H. W. (1974) in: Methods in Molecular Biology; DNA Replication and Biosynthesis (Wickner, R. B. ed) Vol. 7.
[10] Thomas, M. and Davis, R. W. (1975) J. Mol. Biol. 91, 315–328.
[11] Greene, P. J., Poonian, M. S., Russbaum, A. L., Tobias, L., Garfin, D. E., Boyer, H. W. and Goodman, H. M. (1975) J. Mol. Biol. 99, 237–262.
[12] Modrich, P. and Zabel, D. (1976) J. Biol. Chem. 251, 5866–5874.
[13] Atkinson, A. and Jack, G. W. (1973) Biochim. Biophys. Acta 308, 41–52.
[14] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Holloway, P. W. (1973) Anal. Biochem. 53, 304–308.
[16] Burton, K. (1956) Biochem. J. 62, 315–323.
[17] Millitzer, W. E. (1946) Arch. Biochem. 9, 85–90.
[18] Burgess, R. R. and Jendrisak, J. J. (1975) Biochemistry 14, 4634–4638.
[19] Jendrisak, J. J. and Burgess, R. R. (1975) Biochemistry 14, 4639–4645.
[20] Laemmli, U. K. (1970) Nature 227, 680–685.